

Isolation of procathepsin D from mature cathepsin D by pepstatin affinity chromatography

Autocatalytic proteolysis of the zymogen form of the enzyme

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Procathepsin D is a rapidly processed precursor form of the lysosomal proteinase cathepsin D. The enzymic properties of procathepsin D have been studied by examining the pepstatin-binding characteristics of both the precursor and the mature enzyme. Procathepsin D bound to immobilized pepstatin at 4 °C in pH 3.5 buffer but not in pH 5.3 buffer, whereas mature forms of cathepsin D bound to immobilized pepstatin at both pH values. These characteristics of procathepsin D were exploited to isolate the proenzyme from mature forms and to determine whether activation of the proenzyme is an autocatalytic process. After incubation at 37 °C in pH 3.5 buffer, the proenzyme underwent pepstatin-inhibitable proteolysis resulting in a dramatically increased affinity of purified procathepsin D for pepstatin at pH 5.3. The low concentration of enzyme used in these studies suggests that procathepsin D cleavage to single-chain cathepsin D may occur via a unimolecular mechanism.

INTRODUCTION

The lysosomal aspartic proteinase cathepsin D (EC 3.4.23.5) is synthesized as a preproenzyme that undergoes several proteolytic cleavages during biosynthesis to produce the mature enzyme (for review see Conner *et al.*, 1987). The first cleavage is co-translational and removes the signal peptide to yield procathepsin D, a presumably inactive glycosylated form of cathepsin D. The proenzyme is processed to remove ultimately 44 amino acid residues from the *N*-terminus, producing an active single-chain molecule with a molecular mass of approx. 44 kDa. This molecule is eventually cleaved in the lysosome into a two-chain enzyme consisting of a 15 kDa *N*-terminal domain and a 30 kDa *C*-terminal domain. This latter cleavage results in the removal of seven amino acid residues between the light chain and the heavy chain (Shewale & Tang, 1984; Faust *et al.*, 1985). Finally, several amino acid residues are removed from the *C*-terminus of the heavy chain (Erickson & Blobel, 1983). In cultured cells most of the procathepsin D is converted into the mature forms, but, however, a small quantity of procathepsin D is secreted into the medium. Even smaller quantities of the mature two-chain enzyme can be detected in the medium, which are correlated with cell lysis and release from lysosomes.

The activities of the various forms of cathepsin D have been examined and both the single-chain and two-chain forms are enzymically active (Sapolsky & Woessner, 1972; Huang *et al.*, 1979). The activity of procathepsin D has been more difficult to assess. Procathepsin D, contained in immunoprecipitates of culture medium, has also been shown to undergo a pH-dependent pepstatin-inhibitable change in molecular mass at pH 3.5 that correlates with an increase in enzymic activity (Hasilik *et al.*, 1982). These observations, taken together with the high degree of amino acid sequence similarity of procathepsin D and the other aspartic proteinases, suggest that procathepsin D is an inactive precursor of cathepsin D. However, owing to the limited quantities of the

enzyme available and the contamination with mature forms, it has not been possible to examine the mechanism of activation of the proenzyme. To investigate further the enzymic characteristics of procathepsin D and its functional analogy to pepsinogen, I have now examined the ability of this form of the protein to bind the peptide inhibitor pepstatin and have exploited these characteristics to purify procathepsin D from cathepsin D and examine activation in the absence of mature enzyme.

EXPERIMENTAL

Cell growth and metabolic labelling

Pig kidney (PK15) cells (A.T.C.C. CCL56) were maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum and radiolabelled with [³⁵S]methionine (800–1000 Ci/mmol) as previously described (Erickson *et al.*, 1981).

Affinity chromatography

Pepstatinyl-agarose was prepared as described by Huang *et al.* (1979). All operations were performed at 4 °C and all tubes and pipettes were pre-coated with a 100 µg/ml solution of gelatin (60 Bloom; Sigma Chemical Co.). For affinity chromatography of cellular material, metabolically labelled cells were washed three times in 0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4, solubilized in 0.2% Triton X-100/5 mM-EDTA/5 mM-Tris/HCl buffer, pH 8.3, and centrifuged at 12000 *g* for 10 min. The supernatant was diluted with 2 vol. of either 0.2% (v/v) Triton X-100/0.7 M-NaCl/0.2 M-sodium formate buffer, pH 3.5, or 0.2% (v/v) Triton X-100/0.7 M-NaCl/0.2 M-sodium acetate buffer, pH 5.3. After addition of bovine serum albumin to 1 mg/ml, supernatants were incubated with pepstatinyl-agarose beads for 1 h at 4 °C. The beads were washed five times with 0.1% Triton X-100/0.4 M-NaCl in buffers at the concentration and pH used for binding. Bound protein was eluted by incubation in 0.02% Triton X-100/0.4 M-NaCl/20 mM-Tris/HCl buffer, pH 8.3, and then freeze-

dried and washed with 10% (w/v) trichloroacetic acid. All other cell-derived fractions were precipitated with 10% trichloroacetic acid. For immunoprecipitation, samples were solubilized by heating to 100 °C in 0.5% SDS/0.1 M-NaCl/2 mM-EDTA/50 mM-triethanolamine and analysed by immunoprecipitation with rabbit anti-(pig cathepsin D heavy chain) antibodies as described previously (Erickson *et al.*, 1981).

For affinity chromatography of secreted material and activation of the eluted protein, culture medium was collected after radiolabelling and clarified of cells and debris by centrifugation at 12000 *g* for 10 min. The medium was adjusted to 0.1% Triton X-100, 0.4 M-NaCl and either 0.1 M-sodium formate buffer, pH 3.5, or sodium acetate buffer, pH 3.5, and then applied to pepstatinyl-agarose columns. Columns were washed with 10–20 vol. of the wash buffers described above and eluted as described above for cellular material. Endoglycosidase H treatments, gel electrophoresis and fluorography were as described previously (Erickson *et al.*, 1981).

Materials

[³⁵S]Methionine (800–1000 Ci/mmol) was purchased from Du Pont NEN Research Products. All tissue-culture media and components were obtained from Grand Island Biological Co. Pepstatin was purchased from Sigma Chemical Co. Aminohexyl-agarose, dicyclohexylcarbodi-imide and *N*-hydroxysuccinimide were purchased from Pierce Chemical Co.

RESULTS AND DISCUSSION

Separation of procathepsin D and mature cathepsin D by pepstatin affinity chromatography

Affinity chromatography has been extensively used to purify mature cathepsin D, as well as other aspartic proteinases, from tissue (see, e.g., Takahashi & Tang, 1981). In addition, pepsinogen has been shown to bind pepstatin, but only at a pH below that necessary for pepsin binding (Marciniszyn *et al.*, 1976). Pepstatin also inhibits the autocatalytic conversion of pepsinogen into pepsin (Marciniszyn *et al.*, 1976). To evaluate the binding of the procathepsin D to pepstatin, pepstatinyl-agarose affinity chromatography was employed with extracts of radiolabelled culture cells that contained the three major forms of the enzyme, procathepsin D, single-chain cathepsin D and two-chain cathepsin D. Non-denaturing-detergent extracts of radiolabelled PK15 cells were mixed with pepstatinyl-agarose at 4 °C in buffers at either pH 5.3 or pH 3.5. After washing at either pH 5.3 or pH 3.5 and elution with pH 8.3 buffer, the various fractions were immunoprecipitated with anti-(cathepsin D heavy chain) antibodies and analysed by gel electrophoresis and fluorography. Procathepsin D bound pepstatin only at pH 3.5, whereas single-chain and two-chain cathepsin D bound pepstatin at pH 3.5 and pH 5.3 (Fig. 1, compare lanes 3 and 4 with lanes 8 and 9). Pre-incubation of the extract with soluble pepstatin abrogated the binding of all forms of the enzyme at pH 3.5 (Fig. 1, compare lanes 6 and 7 with lanes 8 and 9). Incubation of the extracts at 4 °C in buffers at pH 5.3 or pH 3.5 for the duration of the experiment had no effect on the electrophoretic profiles of the immunoprecipitable enzyme (compare lanes 1, 2 and 5). These data indicated that procathepsin D can be distinguished from single-chain and two-chain cathepsin

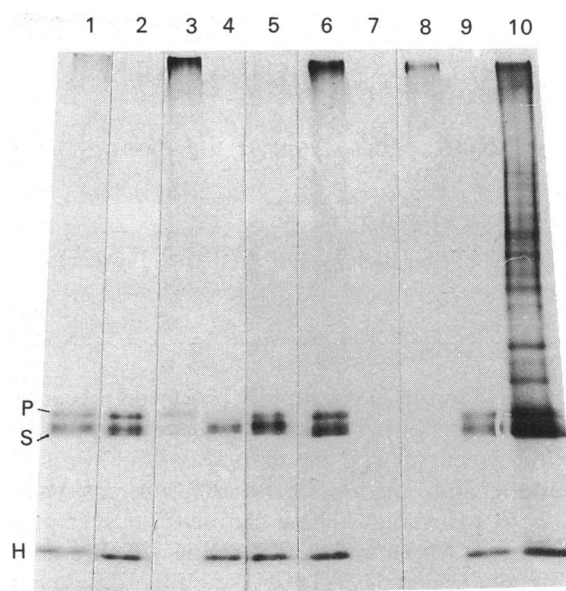


Fig. 1. Pepstatin-binding characteristics of procathepsin D and mature cathepsin D

Ten 100 mm dishes of PK15 cells were incubated for 4 h with [³⁵S]methionine (250 µCi/ml) so that all forms of the enzyme would be labelled. After detergent extraction, equivalent volumes of the extract were either incubated at 4 °C for the duration of the experiment in buffers of different pH or were mixed with pepstatinyl-agarose beads at 4 °C in buffers at different pH in the presence or in the absence of soluble pepstatin. Beads were washed extensively in the binding buffers and eluted in pH 8.3 buffer. Samples were then immunoprecipitated, analysed by electrophoresis on an SDS/10–15%-gradient-polyacrylamide gel and fluorographed. Lane 1 is a control immunoprecipitate of a detergent extract equivalent to those seen in the remainder of the lanes. Lanes 2 and 5 contain fractions that were incubated at pH 5.3 and pH 3.5 respectively for the duration of the experiment and not applied to pepstatinyl-agarose columns to control for endogenous proteolysis. Lanes 3 and 4 are non-bound and bound fractions of extracts applied to pepstatinyl-agarose in pH 5.3 buffer respectively. Lanes 6 and 7 are non-bound and bound fractions respectively of extracts pre-incubated in 0.1 µM soluble pepstatin in pH 3.5 buffer followed by application to pepstatinyl-agarose in pH 3.5 buffer. Lanes 8 and 9 are non-bound and bound fractions of extracts applied to pepstatinyl-agarose at pH 3.5 respectively. Lane 10 is a non-immunoprecipitated sample equivalent to lane 9. P indicates procathepsin D, S indicates single-chain cathepsin D, and H indicates cathepsin D heavy chain.

D on the basis of pH-dependent pepstatin binding at 4 °C, and provided a basis for the purification of small quantities of procathepsin D from other forms of the enzyme. Although the extremely limited availability of procathepsin D required a different approach, these results indicated that the inhibitor-binding properties of procathepsin D were identical with those observed for pepsinogen and pepsin (Marciniszyn *et al.*, 1976).

Autoproteolysis of procathepsin D in the absence of mature enzyme

Procathepsin D, immunoprecipitated from conditioned medium under non-denaturing conditions, has

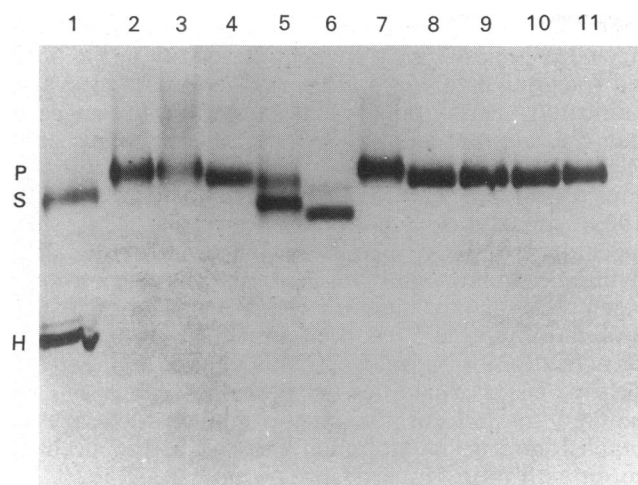


Fig. 2. Endoglycosidase H treatment of procathepsin D and peptide-inhibitor-sensitivity of procathepsin D autoproteolysis

Culture medium, conditioned by cells growing in [35 S]methionine for 12 h, was subjected to two cycles of pepstatinyl-agarose chromatography. The non-bound fraction after the first cycle of chromatography at pH 5.3 was applied to a second column at pH 3.5. Material that bound to the second column was eluted in pH 8.3 buffer. Samples were freeze-dried and washed with 10% trichloroacetic acid, analysed by electrophoresis on an SDS/12%-polyacrylamide gel and fluorographed. Lane 1 contains cathepsin D isolated by pepstatinyl-agarose chromatography at pH 5.3 from cells that were pulsed and chased so that only mature forms of the enzyme would be labelled. The bound and eluted fraction obtained from the second cycle of pepstatinyl-agarose chromatography of culture medium without any post-column treatment is shown in lane 2. Fractions equivalent to that used for lane 2 were incubated in different pH buffers, with or without proteinase inhibitors. Lane 3 was a fraction incubated in pH 8.3 buffer, and lane 4 was a fraction incubated in pH 3.5 buffer. Lanes 5 and 6 are identical with lanes 3 and 4 but the fractions received endoglycosidase treatment after incubation. Lanes 7–11 contain fractions that were incubated in pH 3.5 buffer with various proteinase inhibitors: lane 7, 0.1 μ g of pepstatin/ml (1 μ M); lane 8, 1 μ g of chymostatin/ml; lane 9, 1 μ g of leupeptin/ml; lane 10, 0.04% dimethyl sulphoxide. P indicates procathepsin D, S indicates single-chain cathepsin D, and H indicates cathepsin D heavy chain.

been previously shown to undergo an acid pH-dependent pepstatin-inhibitable conversion into a more rapidly migrating form (Hasilik *et al.*, 1982). To document that this cleavage is due only to autoproteolysis by procathepsin D and not due to contaminating mature enzyme, the pH-dependent pepstatin binding of procathepsin D was exploited to purify the enzyme from possible contaminating mature forms of cathepsin D as well as any other impurities. Medium was collected from radiolabelled cultures of PK15 cells and subjected to two sequential rounds of pepstatin affinity chromatography. After acidification to pH 5.3 and passage through the pepstatinyl-agarose to remove mature cathepsin D, the unbound material was further acidified to pH 3.5 and re-applied to a second pepstatinyl-agarose column. After

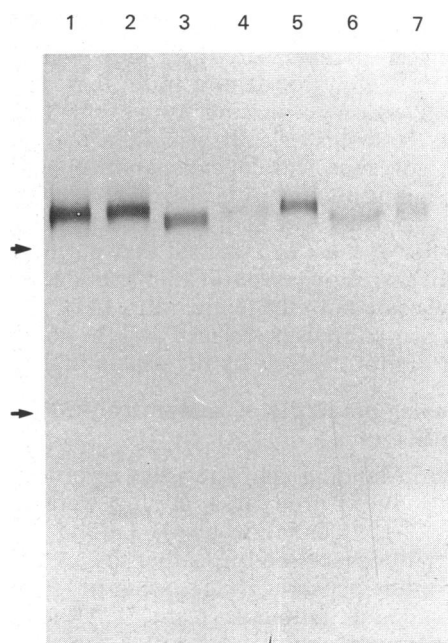


Fig. 3. Effects of autoproteolysis on the pepstatin-binding characteristics of secreted procathepsin D

Procathepsin D, prepared as described in Fig. 2, was either not treated (lane 1), or incubated for 30 min at 37 °C in pH 8.3 buffer (lane 2) or pH 3.5 buffer (lane 3). Samples equivalent to those in lanes 2 and 3 were adjusted to pH 5.3 and rechromatographed on pepstatinyl-agarose. Lanes 4 and 5 are bound and non-bound fractions respectively after pretreatment at pH 8.3. Lanes 6 and 7 show bound and non-bound fractions respectively after pretreatment at pH 3.5. Samples were then analysed by electrophoresis on an SDS/12%-polyacrylamide gel and fluorographed. Arrows show the expected migration of single-chain and heavy-chain cathepsin D (see Fig. 2).

washing at pH 3.5 and elution with pH 8.0 buffer, the final column eluate appeared to contain only procathepsin D (Fig. 2, lane 2). Incubation of this eluate at 37 °C in pH 3.5 buffer demonstrated an acid pH-dependent conversion of procathepsin D into a form with a lower apparent molecular mass (compare lanes 2, 3 and 4). The apparent molecular mass of this material, although less than that of the proenzyme, was greater than that of the single-chain enzyme. A similar incomplete cleavage has been seen after autocatalytic proteolysis of pepsinogen in the presence of pepstatin (e.g. Dykes & Kay, 1976), and was reported for procathepsin D by Hasilik *et al.* (1982).

Post-incubation treatment of these samples with endoglycosidase H to remove *N*-linked oligosaccharides confirmed that the change in mobility after incubation in acid was not due to removal of carbohydrate by contaminating glycosidases (Fig. 2, compare lanes 5 and 6). As shown in previous studies (Erickson *et al.*, 1981), a small amount of endoglycosidase H-resistant forms was found in the proenzyme secreted from cultured cells (Fig. 2, lanes 5 and 6). The conversion of procathepsin D into a more rapidly migrating form was sensitive to pepstatin (Fig. 2, lane 7). Neither leupeptin, nor chymostatin nor dimethyl sulphoxide, the solvent used to solubilize pepstatin, has any inhibitory qualities (Fig. 2, lanes 8–10),

which demonstrated the specificity of the pepstatin inhibition. These data extend the results reported by Hasilik *et al.* (1982) to demonstrate that the acid-dependent conversion was not the result of small amounts of active mature forms of cathepsin D in the immunoprecipitates. Nor is the acid-dependent conversion due to removal of significant quantities of oligosaccharide. In view of this autoproteolytic conversion of the proenzyme in pH 3.5 buffer, it must be assumed that purification of the intact proenzyme by pepstatin affinity under identical conditions relies on both the temperature (4 °C) at which the isolation procedure is performed and the competitive inhibition of autoproteolysis by the pepstatinyl-agarose.

Pepstatin-binding properties of autoproteolysed procathepsin D

The pepstatin-binding characteristics of procathepsin D after acid-induced proteolysis *in vitro* were changed dramatically. After incubation at pH 3.5 and 37 °C for 30 min, partially processed procathepsin D now was capable of binding pepstatinyl-agarose at pH 5.3 (Fig. 3, compare lanes 4 and 5 with lanes 6 and 7). Although only a barely detectable quantity of procathepsin D was adsorbed at pH 5.3 without acid pre-incubation, after acid pre-incubation most of the cleaved procathepsin D was capable of binding pepstatinyl-agarose at the higher pH. These data strongly suggested that the pepstatin-binding characteristics of the converted procathepsin D more closely resembled those of the mature enzyme. Pepstatin has been shown to be an active-site titrant of cathepsin D (Knight & Barrett, 1976), and thus the change in pepstatin-binding characteristics of procathepsin D after proteolysis probably represents production of an active form of the enzyme. The ability to re-bind pepstatin indicates that the failure of the intermediate form of cathepsin D to proceed autocatalytically to the single-chain form, as is seen with pepsinogen intermediates, is not the result of denaturation at low protein concentration and subsequent loss of active-site conformation. Instead, this intermediate may represent a previously unreported form of the enzyme *in vivo* that requires another proteinase for full maturation to single-chain cathepsin D.

Since procathepsin D has a short half-life in the cell and only a portion of the enzyme is secreted (Erickson *et al.*, 1981), chemically measurable quantities of procathepsin D cannot be isolated without the use of extremely large-scale cultures. These experiments therefore utilized exceedingly low concentrations (< 1 nM) of

radiolabelled enzyme. These concentration of enzyme precluded accurate measurement of activity in the purified fractions before and after acid-dependent cleavage. Autocatalytic processing at these concentrations suggests that the enzyme activation described here may have occurred by a unimolecular mechanism. The relatively slow appearance of activity reported by Hasilik *et al.* (1982) suggests a bimolecular activation. This slow appearance of activity could be the result of the large quantity of immunoglobulin present in the preparations, which acts as an inhibitor of the activation. Such inhibition by protein has been previously demonstrated for activation of pepsinogen (Marciniszyn *et al.*, 1976). Clearly, larger quantities of procathepsin D will be required to perform the kinetic studies necessary to establish directly unimolecular cleavage as the mechanism of activation.

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